METABOLIC ENGINEERING OF NITROGEN ASSIMILATION IN TRANSGENIC PLANTS:

Ectopic overexpression of Fd-GOGAT GLU1 gene in *Arabidopsis thaliana* results in enhanced growth phenotype.

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INTRODUCTION

Assimilation of inorganic nitrogen and carbon into organic matter is a pivotal process for plant growth, as it has marked effects on plant productivity, biomass, and crop yield (Lawlor *et al.,* 1989; Mattsson *et al.,* 1991). Nitrogen deprivation in plants has been shown to cause a number of metabolic deficiencies in plants including reduced photosynthetic capacity and carboxylation efficiency (Delgado *et al.,* 1994). Thus, nitrogen is a rate-limiting element in plant growth (Mattsson *et al.*, 1991).

The first step in the assimilation of inorganic nitrogen (ammonium) into organic nitrogen (amino acids) is the conversion of ammonium into glutamine and glutamate by the coordinated actions of the GS and GOGAT enzymes. Assimilation of nitrogen into glutamine and glutamate is then followed by the biosynthesis of aspartate by AspAT and asparagine by the enzyme AS. However, different isoenzymes in the pathway of nitrogen assimilation may play different roles depending on both environmental conditions (*e.g.* light and metabolites) and different tissue types. Under light growth conditions where plants possess a high carbon-to-nitrogen metabolite ratio, chloroplastic GS2/GLN2 and Fd-GOGAT/GLU1 are the major isoenzymes responsible for the reassimilation of photorespiratory ammonium in leaves where they might also be involved in primary assimilation of ammonium. Nitrogen assimilated by the GS/GOGAT cycle into glutamate and glutamine, can follow two major routes: (a) these amino acids can serve as the major nitrogen donors in various anabolic reactions and/or (b) they can be channeled

into other nitrogen carriers such as aspartate and asparagine, by the action of AspAT and AS/ASN1, respectively (see Fig. 1).

As the GS/GOGAT cycle is first step in the assimilation of inorganic nitrogen into organic form, we postulated that it might be possible to increase yield or improve crop quality by the molecular or genetic manipulation of these genes involved in nitrogen assimilation. We previously showed that the ectopic overexpression of GS genes in transgenic plants leads to enhanced growth properties. Here we report the data resulting from the ectopic overexpression of a gene for Fd-GOGAT (GLU1). Our initial results indicate that the overexpression of Fd-GOGAT GLU1 gene results in an ameliorated growth phenotype in transformed *Arabidopsis thaliana* plants. This conclusion is supported by 1) the correlation between the increased growth phenotype and the increased levels of Fd-GOGAT mRNA expression and 2) the increased growth rate, as judge by root length, of transgenic plants overexpressing Fd-GOGAT grown side-by-side with wild-type plants in media supplemented with different concentrations of inorganic nitrogen (ammonium and nitrate).

METHODS

ISOLATION OF Fd-GOGAT cDNA

(K.Coschigano, R.Melo-Oliveira and G.Coruzzi, unpublished)

Thirteen partial Fd-GOGAT clones were isolated from an Arabidopsis cDNA library made from light-grown plant tissue using a 1 kb PCR product obtained from the pea Fd-GOGAT gene (Coruzzi and McGrath, unpublished results). Hybridization occurred overnight in 5 X Denhardt's, 6 X SSPE, 1 mM EDTA, 0.1 % SDS and 0.05 mg/ml salmon sperm DNA at 50°C. Washes were performed to a final stringency of 1 hr at 42°C in 0.1 X SSC, 0.1% SDS. Clones were obtained in the vector Bluescript SK^- by *in vivo* excision of the lambda ZAP clones (Stratagene). Longer cDNA clones were obtained by screening a library made from etiolated seedlings using clones obtained from the first library as probes. Full-length cDNA sequence at the 5' end was generated from cDNA fragments obtained using the Gibco BRL 5' RACE system (both *GLU1*). Products

were subcloned using the pCR-Script kit. Sequencing was performed using the TN1000 Advanced Nested Set Technology (Gold BioTechnology) and the dideoxy sequencing method. Twelve cDNA clones identical to each other in the region sequenced were named pAtGLU1.

Since none of the cDNA clones were full-length (greater than 5 kb mRNA), the complete Fd-GOGAT GLU1 cDNA sequence described is a composite generated using additionally isolated cDNAs and 5'RACE products. *GLU1* cDNA encode an amino terminal extension to the mature protein, characteristic of a chloroplast transit peptide (high serine/threonine content and net positive charge; Keegstra *et al.*, 1989). The Arabidopsis GOGAT GLU1 protein encoded by *GLU1* is more similar to the Fd-GOGAT sequence of maize (79% and 76% identity, respectively) than to the NADH-GOGAT sequence of alfalfa (41% identity for each). Furthermore, the Fd-GOGAT *GLU1* gene of Arabidopsis and maize are lacking the additional 550 amino acids at the carboxy terminus of NADH-GOGAT from alfalfa which encodes the NADH binding domain (Gregerson *et al.*, 1993). Thus, it appears that the Arabidopsis *GLU1* encode an isoenzyme of the Fd-GOGAT family.

Construction of the 35SCaMV-GLU1 (35S-Fd-GOGAT) plant expression vector.

The full-length Fd-GOGAT GLU1 cDNA was excised from the vector Bluescript SK^- by digestion with the endonuclease EcoRI. After purification the Fd-GOGAT GLU1 cDNA fragment was made blunt-end by Klenow fill-in of recessive ends and subcloned into the blunt-ended XbaI site of a pBI 101-based plant expression vector (pTEV7) to generate the pCaMV-GLU1 construct (see fig.2).

Transformation of *Arabidospsis thaliana* plants with the pCaMV-GLU1 construct.

Following introduction of the 35SCaMV-Fd-GOGAT (35S-GLU1) construct into the *Agrobacterium tumefasciens* strain GV3101 by electroporation, this strain was used to transform *Arabidopsis thaliana* plants by vacuum-infiltration. The transformed plants were grown to full maturity in soil, seeds were collected and sown in MS media supplemented with kanamycin. Kan^r individuals were transferred to soil and seeds were collected from each separate individual for segregation and gene expression analysis. In

parallel, a set of *Arabidopsis thaliana* plants was transformed with the pCaMV vector alone and used as "wild-type" control in the root growth experiments outlined below.

RESULTS: Ectopic expression of Fd-GOGAT in transgenic plants

Arabidopsis thaliana t_1 plants overexpressing Fd-GOGAT GLU1 gene show an improved growth phenotype. In our first round of screening, 70 kan^r plants transformed with the 35S-Fd-GOGAT construct (t_1 generation) were transplanted to soil. Out of 67 survivors, visual inspection revealed two distinct classes of transgenic plants. The first class represented by 5 individuals (GLU1-102, GLU1-103, GLU1-104, GLU1-105 and GLU1-106), all displayed similar characteristics as follows: 1) a general increase in size with regard to both leaf area and stem length and 2) a "greener" appearance when compared to the surrounding plants of the same age (fig.3A-3E; see photographs attached). The second class of Fd-GOGAT transformants (62 individuals) represented by the line GLU1-100 show a characteristic leaf chlorosis. Based on these growth phenotypes of these two classes of plants, we proposed that the the first class of transformants ameliorated growth phenotype (represented herein by lines GLU1-104 and GLU1-105) are Fd-GOGAT overexpressing plants, and that the second class of plants (represented by line GLU1-100) are chlorotic and co-supressed for the Fd-GOGAT gene. It is noteworthy that a similar chlorotic phenotype has been observed for Fd-GOGAT mutants of Arabidopsis (Somerville and Ogren, 1980).

Analysis of Fd-GOGAT gene expression in Fd-GOGAT/GLU1 transgenic Arabidopsis plants.

Levels of Fd-GOGAT mRNA were monitored in control plants (transformed with vector alone), putative cosuppressed Fd-GOGAT plants (GLU1-100), and Fd-GOGAT overexpressing plants (GLU1-104, 105) (Fig. 4). Seeds were sown in MS media supplemented with kanamycin. After 14 days, tissue was collected and Northern blot experiments were performed (fig.4). Probes were PCR-generated single-stranded DNA fragments and the Fd-GOGAT/GLU1 probe is a gene-specific probe. An *Arabidospsis thaliana* βATPase probe was used as an internal control. As anticipated, levels of Fd-GOGAT mRNA are reduced in the cosuppressed line GLU1-100 (Fig. 4, lane 2) and

induced in the overexpressing lines GLU1-104 and GLU1-105 (Fig. 4, lanes 3 & 4) when compared to the control line (fig.4, lane 1).

Analysis of the growth rate of Fd-GOGAT/GLU1 transgenic Arabidopsis plants. For the assays designed to assess growth rate as a function of root length in the 35S-Fd-GOGAT transgenic lines (Fig. 5), seeds are sown side-by-side with controls (transformed with vector alone) on square tissue culture plates containing either on Murashige and Skoog (MS) medium (with no inorganic nitrogen source) (Fig. 6A) or MS medium supplemented with 0.4 mM nitrate and 0.2 mM ammonium (Fig. 6B) or 4 mM nitrate and 2 mM ammonium (Fig. 6C). Plates are oriented vertically so root length is measured as function of growth rate. After the incubation period, root length of both the wild-type and Fd-GOGAT transformants were measured and compared (fig.5). We observed that the two overexpressing lines GLU1-104 and GLU1-105 showed increased root length when compared to the wild-type plants regardless of the concentration of inorganic nitrogen in the media (Figs. 5A and Fig. 6A-C). By contrast, the line co-suppressed for Fd-GOGAT (GLU1-100) demonstrated a retarded growth phenotype when grown under all nitrogen regimes tested (Fig. 5B).

Conclusions

We have created transgenic Arabidopsis plants that ectopically overexpress an Arabidopsis gene for Fd-GOGAT (GLU1). The transgenic plants fall into two classes: Fd-GOGAT overexpressing plants (e.g. GLU1-104 and GLU1-105) that show increases in Fd-GOGAT mRNA and an accompanying enhanced growth phenotype. The second class of plants appear to be cosuppressed for Fd-GOGAT (e.g. GLU1-100) based on reductions in level of Fd-GOGAT mRNA and chlorotic growth phenotype. These studies extend our previous work which showed that the ectopic expression of GS genes in transgenic tobacco lead to an enhanced growth phenotype. Thus, our studies show that maniuplation of two genes in the nitrogen assimilatory pathway (shown in Fig. 1) GS and GOGAT from two different species (pea and Arabidopsis, respectively) when overexpressed in two different transgenic species (tobacco and Arabidopsis) results in an enhanced growth phenotype.

Bibliography

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FIGURE LEGENDS:

Figure 1. Assimilation of nitrogen into the nitrogen transport amino acids glutamine, glutamate, asparagine and aspartate. The first step in the assimilation of inorganic nitrogen (ammonium) into organic nitrogen (amino acids) is the conversion of ammonium into glutamine and glutamate by the coordinated actions of the GS and GOGAT enzymes. Assimilation of nitrogen into glutamine and glutamate is then followed by the biosynthesis of aspartate by AspAT and asparagine by the enzyme AS. The two possible functions of GDH, catabolic with the production of ammonium and 2-oxoglutarate and anabolic represented by the biosynthesis of glutamate, are also represented.

Figure 2. Construction of pCaMV-Fd-GOGAT. The cDNA coding for the *Arabidopsis thaliana* Fd-GOGAT GLU1 gene was cloned as described (Coschigano et al, submitted). The full length cDNA GLU1 clone was introduced downstream from the CaMV 35S promoter/Kan^r into the vector pTEV (Brears and Coruzzi). This construct was introduced into *A.thaliana* plants by vacuum infiltration.

Figure 3. Improved Growth Phenotype of *Arabidopsis thaliana* t_1 plants overexpressing Fd-GOGAT GLU1 gene. Seeds of*Arabidopsis thaliana* plants transformed with the pCaMV-Fd-GOGAT construct were selected in kanamycin containing MS media and transferred to soil. Pictures show 55 day-old plants (panels A-E).

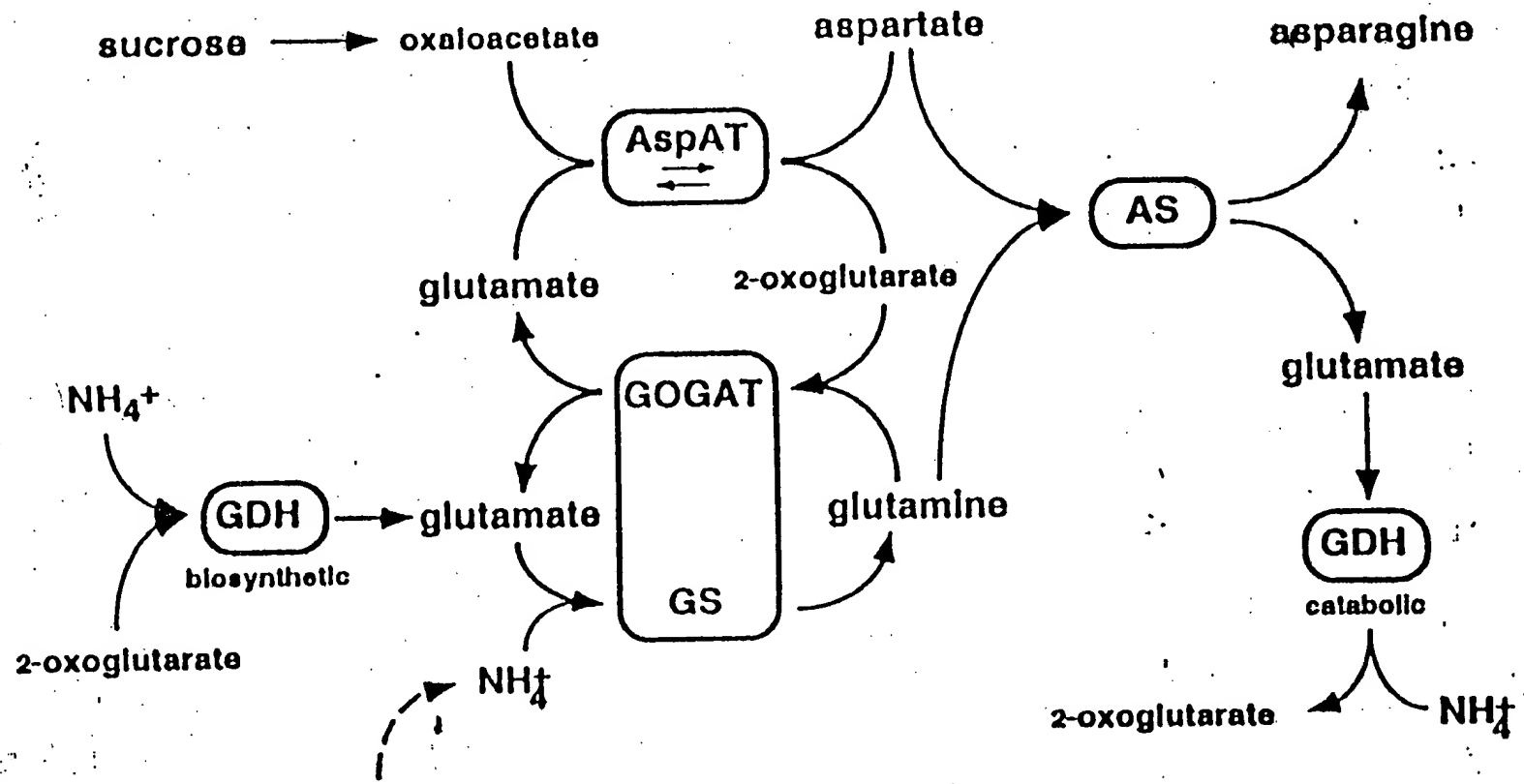
Figure 4. Northern analysis of mRNA in transgenic plants shows an increase in Fd-GOGAT mRNA in 35S-Fd-GOGAT overexpressing plants. Northern blot analysis of total RNA extracted from either wild-type plants or t_1 populations. Lane 1, wild-type *Arabidopsis thaliana* (transformed with vector alone); lane 2, GLU1-100 Fd-GOGAT transformed line (cosuppressed) showing a decreased level of mRNA accumulation compared to wt plants; lanes 4 and 5, GLU1-104 and GLU1-105, (overexpressor)

respectively, showing their increased level of expression of GOGAT mRNA. The probes used were PCR-generated single-stranded DNA fragments and the Fd-GOGAT/GLU1 probe is a 5' gene-specific probe. The *Arabidospsis thaliana* βATPase probe was used as an internal control.

Figure 5. Plants which overexpress Fd-GOGAT/GLU-1mRNA show an enhanced growth rate compared to wild-type plants. For the assays designed to assess growth rate in Fd-GOGAT overexpressing lines, seeds are sown aligned side-by-side on square tissue culture plates containing either on Murashige and Skoog (MS) medium containing no inorganic nitrogen cource, or on MS medium supplemented with 0.4 mM nitrate /0.2 mM ammonium or 4 mM nitrate/2 mM ammonium (see also fig.6). Plates are oriented vertically so root length is measured as function of growth rate. After the incubation period, root length of both the wild-type and Fd-GOGAT transformants were measured and compared. (A) Comparative growth of the overexpressor line GLU1-104 with wild-type Arabidopsis plants. (B) Comparative growth of the co-supressed line GLU1-100 with wild-type Arabidopsis plants.

Figure 6. Plants which overexpress Fd-GOGAT mRNA (GLU1-104, Glu1-105) transgenic plants show enhanced growth compare to wild-type. For the assays designed to assess growth rate in Fd-GOGAT overexpressing lines, seeds are sown aligned side-by-side on square tissue culture plates containing either on Murashige and Skoog (MS) medium containing no inorganic nitrogen source (Panel A), or on MS medium supplemented with 0.4 mM nitrate /0.2 mM ammonium (Panel B); or 4 mM nitrate/2 mM ammonium (Panel C). Plates are oriented vertically so root length is measured as function of growth rate. After the incubation period, root length of both the wild-type and Fd-GOGAT transformants were measured and compared. The enhanced growth phenotype of two Fd-GOGAT overexpressing lines are shown GLU1-104 (A) and GLU1-105 (B), compared to wild-type transformed with vector alone.

Figure 1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oxaloacetate
aspartate
asparagine
AspAT
AS
glutamate
2-oxoglutarate
glutamate
NH_4^+
GOGAT
GDH
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glutamine
GS
GDH
catabolic
2-oxoglutarate
NH_4^+
2-oxoglutarate
NH_4^+

Figure 2

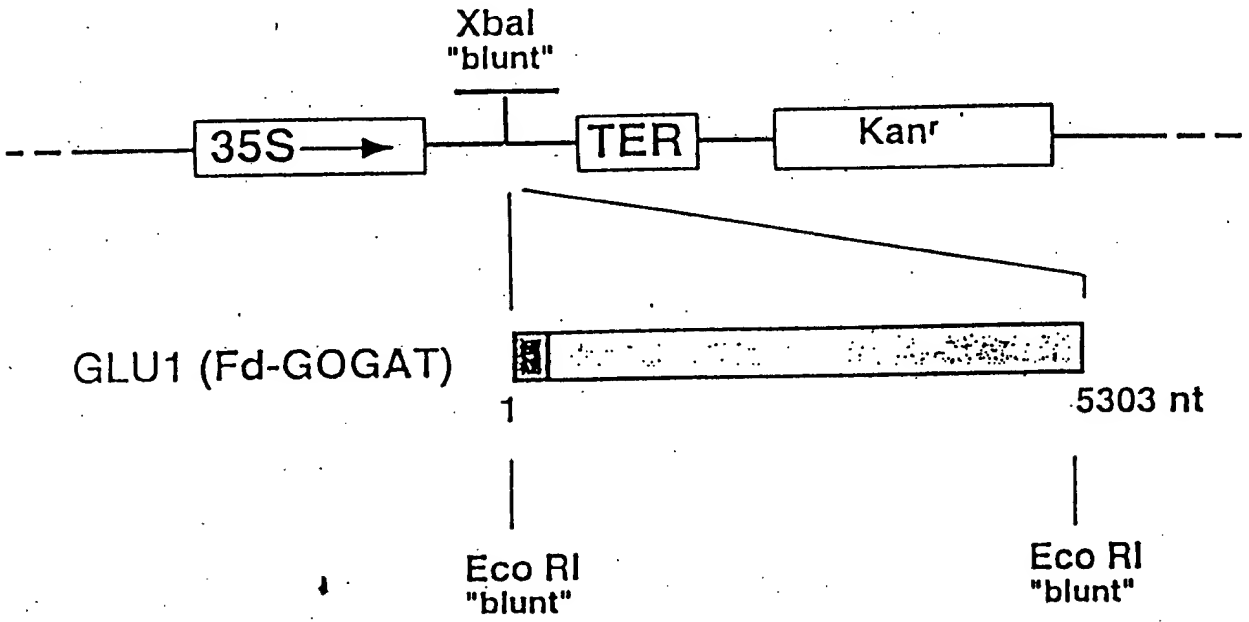
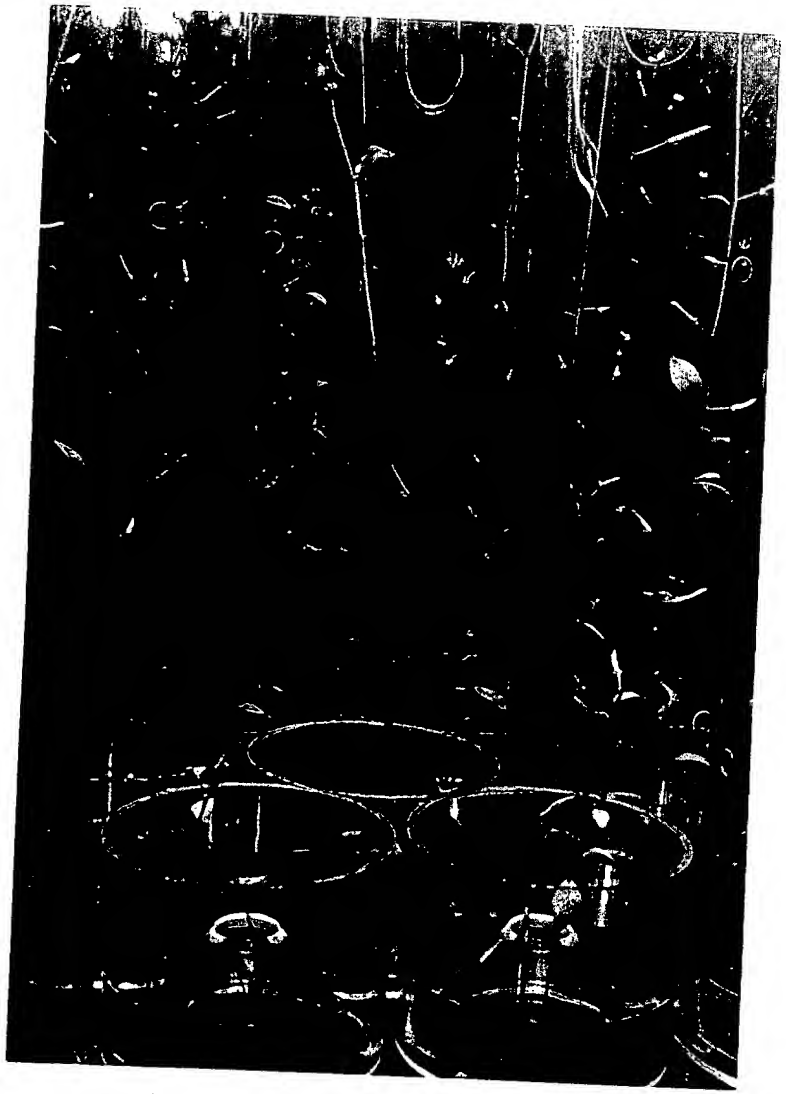

XbaI
"blunt"
35S
TER
Kanr
GLU1 (Fd-GOGAT)
1
5303 nt
Eco RI
"blunt"
Eco RI
"blunt"

FIG.3



A

B

FIG.3

C

D

E

Figure 4

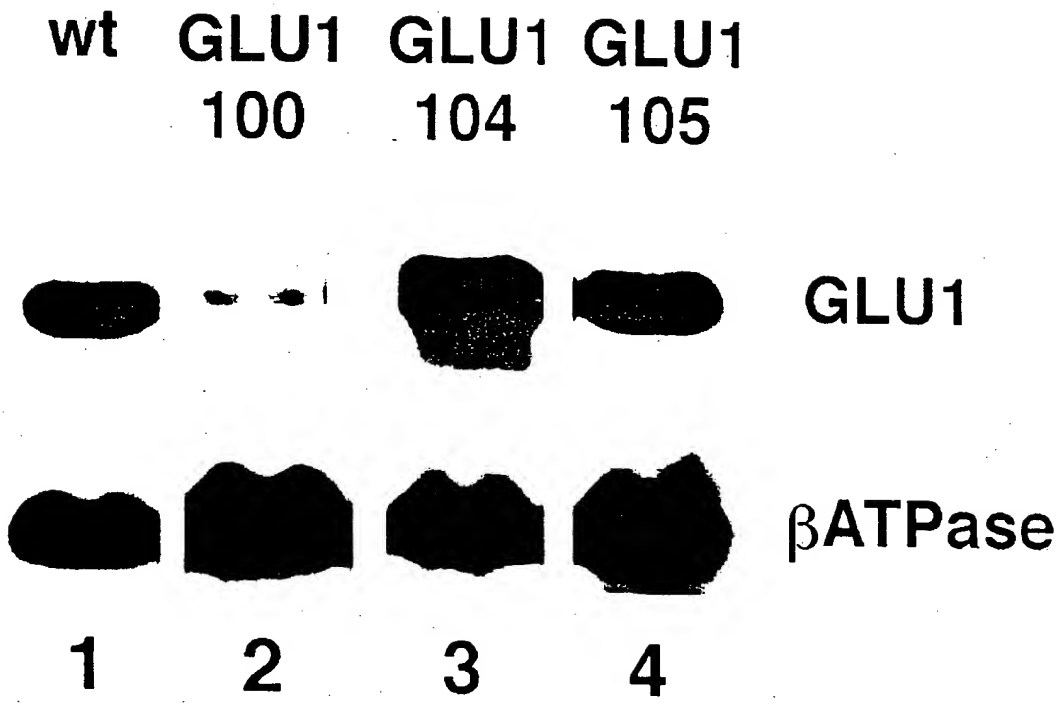
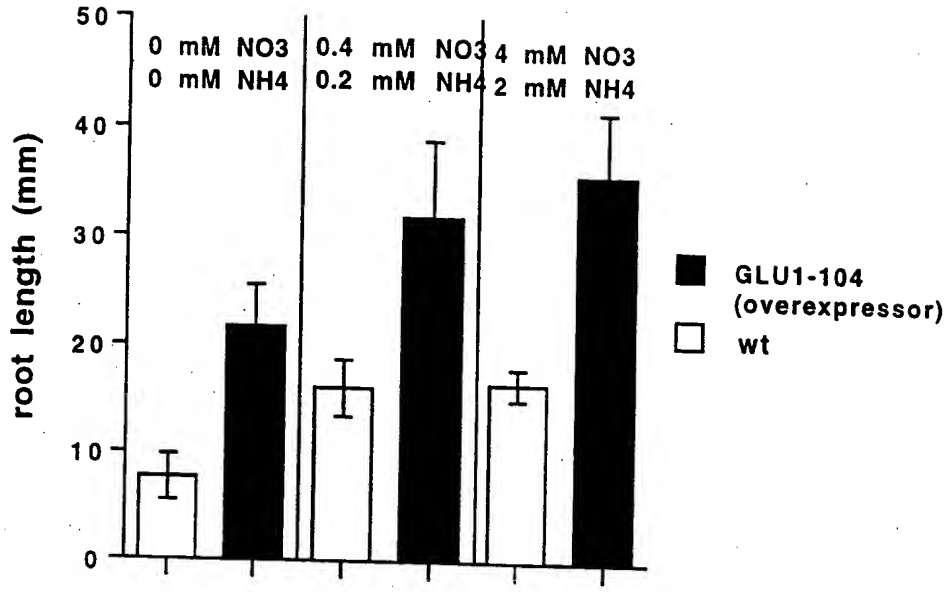


Figure 5

A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40
30
20
10
0
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0 mM NH4
0.4 mM NO3
0.2 mM NH4
4 mM NO3
2 mM NH4
GLU1-104
(overexpressor)
wt

B

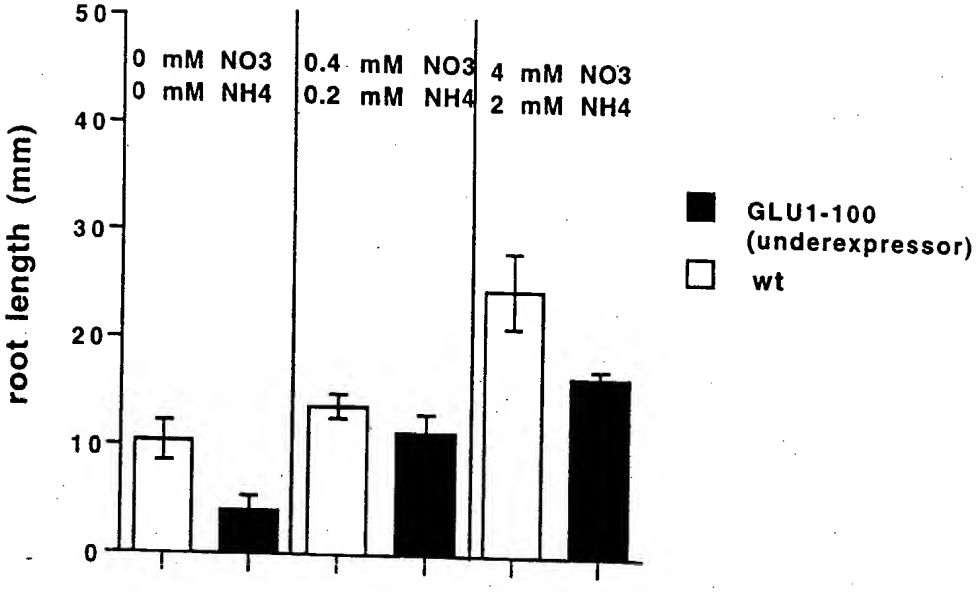
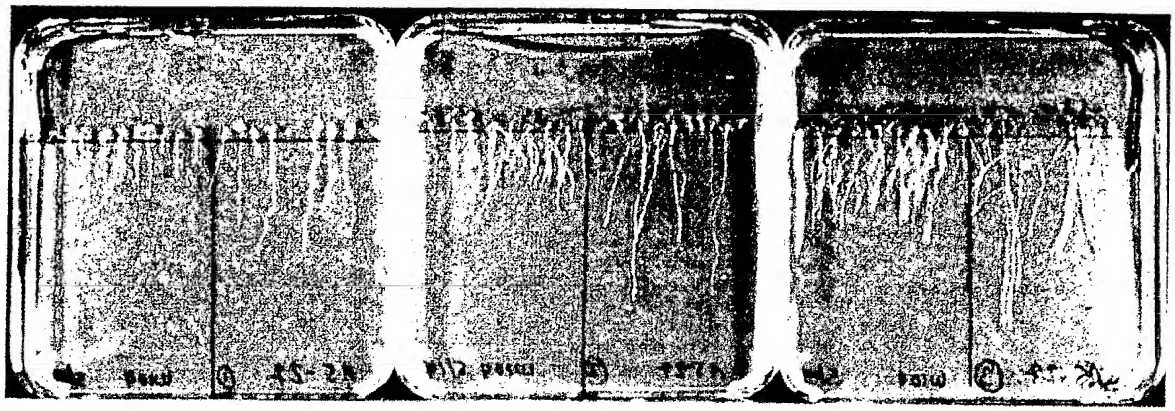

root length (mm)
50
40
30
20
10
0
0 mM NO3
0 mM NH4
0.4 mM NO3
0.2 mM NH4
4 mM NO3
2 mM NH4
GLU1-100
(underexpressor)
wt

Figure 6

A



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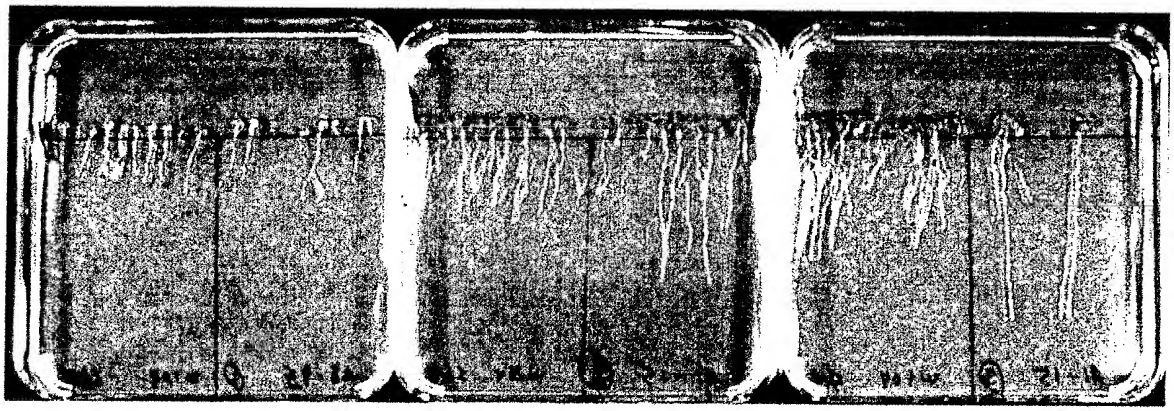
0 mM NH_4 0.2 mM NH_4 2 mM NH_4

B



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0 mM NH_4 0.2 mM NH_4 2 mM NH_4